The measurement of the lactonase activity of paraoxonase-1 in the clinical evaluation of patients with chronic liver impairment

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Abstract

Aims: We investigated the analytical performance of a new assay of the lactonase activity of paraoxonase-1 and its efficacy in the assessment of liver damage.

Design and methods: Serum lactonase activity was determined by the hydrolysis of 5-thiobutyl butyrolactone in 633 healthy individuals and 369 patients with chronic liver disease. Paraoxonase-1, 2, and 3 gene polymorphisms were analyzed by the MassArray™ method.

Results: Linearity was up to 10 U/L. Detection limit was 0.12 U/L. Imprecision was ≤ 17.7%. Lactonase values in our normal population were 5.99 (3.29–13.61) U/L. Lactonase activity showed a lower influence of genetic polymorphisms than the classical assay using paraoxon. Both measurements showed a similar efficiency in testing for liver dysfunction.

Conclusion: We report a reliable assay using a non-toxic substrate for the measurement of serum lactonase activity. The influence of genetic variability is low. The assay could be a useful addition to tests evaluating liver impairment.

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Keywords: Diagnostic accuracy; Lactonase; Liver diseases; Paraoxonase; Reference interval

Introduction

Paraoxonases are a family of three enzymes termed PON1, PON2 and PON3, whose genes are located adjacent to each other on chromosome 7q21–22 [1,2]. All these enzymes have had polymorphisms identified in the coding as well as the promoter regions, and their impact on their respective protein products is the subject of ongoing research [3]. The best known among the paraoxonases is PON1 which acts as an esterase and lactonase catalyzing the hydrolysis of organophosphates and other xenobiotics. It is found in the circulation bound to high-density lipoproteins (HDL) [4–6]. The original function of PON1 was that of a lactonase, and lipophylic lactones constitute its primary substrates [5,7]. PON1 degrades oxidized phospholipids in low-density lipoproteins (LDL) and HDL and, as such, plays a role in the organism’s antioxidant system [8–11]. Alterations in circulating PON1 levels are associated with a variety of diseases involving oxidative stress [12–14].

The liver plays a key role in the synthesis of PON1 [15,16] and chronic liver diseases are associated with increased oxidative stress and lipid peroxidation [17,18]. We have reported [19–21] that serum PON1 activity is decreased in patients with chronic liver impairment, and have suggested that serum PON1 activity measurement may improve the evaluation of liver function in these patients. The potential clinical interest of these observations is high but there are some major
limitations in the current methods for PON1 measurement that restrict its applicability. PON1 activity is usually determined by measuring the hydrolysis of paraoxon, a highly toxic and unstable compound which is not suitable for a high-throughput routine method. Secondly, the interpretation of the physiological significance of a measurement conducted with such an unnatural substrate is questionable. In addition, the enzyme’s activity on paraoxon is strongly influenced by the PON1 genetic polymorphism [4]. For these reasons, substantial improvement in the choice of substrate is mandatory before applying this analysis in the clinical setting.

Recently, a new fast, semi-automated PON1 assay has been reported. The method is based on a chromogenic lactone that resembles the proposed natural lipolactone substrates. Preliminary data suggest that this method is reliable in measuring HDL-associated PON1 [22]. The present study was aimed at: (a) evaluating the analytical performance of a new lactonase activity paraoxonase-1 assay; (b) investigating the influence of genetic variability in a population-based study; (c) investigating the efficacy of the lactonase assay in the assessment of liver damage.

Materials and methods

Study participants

We analyzed samples from a population-based study conducted in our area. Details of this study have been published [23]. The participants were healthy subjects (n=633; 339 women, 294 men; mean age: 45 years, range 18 to 81) of Caucasian ethnic origin from the Mediterranean region of Catalunya. All the volunteers had been invited to attend a clinical examination and to provide a fasting blood sample. There was no clinical or analytical evidence of renal insufficiency, liver damage, neoplasia or neurological disorders.

The patient population consisted of 369 individuals with chronic liver disease (266 men and 103 women; mean age: 47 years, range 20 to 80 years) who had been admitted to one of the participating hospitals for treatment. The etiology of the disease was alcoholic in 178 (48%) and viral in 191 (52%) individuals. Samples for analyses were obtained within 24 h of admission. For the purpose of the present study, patients were segregated into two groups. One group (n=215; 158 men and 57 women) contained those patients with mild-to-moderate liver disease with mild hepatomegaly and/or a moderate increase in serum aminotransferase or γ-glutamyl transferase activities, but with no ultrasound changes that would suggest cirrhosis. The second group (n=154; 108 men and 46 women) contained those patients with liver cirrhosis confirmed by histology examination. The study was approved by the Ethics Committees of the Hospitals involved in the study and all patients and control subjects gave written consent to participation.

Biochemical measurements

Blood samples were collected after an overnight fast into tubes with no anticoagulants to obtain serum, or into tubes with K₂-EDTA for genetic analyses, or into Na₂-citrate tubes for plasma prothrombin time measurements. The analyses were performed immediately, or aliquots of material were stored at −80 °C for subsequent batched analyses. Serum lactonase activity was analyzed by measuring the hydrolysis of 5-thiobutyl butyrolactone (TBBL) as described [22]. All assays were performed on 96-well plates (Nunc™, Roskilde, Denmark) using an automated microplate reader (BioTek Instruments Inc., Winooski, VT, USA). Lactonase activity was measured in an assay reagent containing 1 mmol/L CaCl₂, 0.25 mmol/L TBBL and 0.5 mmol/L 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) in 0.05 mmol/L Tris–HCl buffer (pH =8.0). The change in absorbance was monitored at 412 nm. Activities were expressed as U/L (1 U=1 mmol of TBBL hydrolyzed per minute). The shelf-life of TBBL solution is approximately 2 months and stocks were replenished within this period. Serum paraoxonase activity was determined by measuring the rate of hydrolysis of paraoxon at 410 nm and 37 °C in a 0.05 mmol/L glycine buffer (pH=10.5) with 1 mmol/L CaCl₂ [19]. Activities were expressed as U/L (1 U=1 μmol of paraoxon hydrolyzed per minute). Paraoxon substrate had to be prepared fresh for every batch of measurements. Serum alanine aminotransferase (ALT), and γ-glutamyl transferase (GGT) activities and cholesterol, triglycerides, albumin, bilirubin, apolipoprotein A—I, and HDL–cholesterol concentrations were measured with reagents obtained from Beckman-Coulter in a Synchron Lxi automated analyzer (Beckman-Coulter, Fullerton, CA, USA). Plasma prothrombin time was measured in an ACL 9000 analyzer (Instrumentation Laboratories, Milan, Italy).

Control materials

There are no commercial control materials for use in performance studies of lactonase activity measurement. Instead, we used three pools of sera prepared from samples from the healthy population described above. Sera with the lowest, the highest, and the intermediate lactonase activities were pooled, gently mixed for 2 h at 4 °C and divided into aliquots for storage at −40 °C. The three pools were “Pool A” (3.96 ±0.70 U/L), “Pool B” (5.36 ±0.70 U/L), and “Pool C” (8.00 ±0.94 U/L).

PON1, PON2, and PON3 genotyping

Genomic DNA was obtained from leukocytes (Puregene DNA Isolation reagent set, Gentra Systems Inc., Minneapolis, MN, USA). Selected single nucleotide polymorphisms (SNP’s) were analyzed by the Iplex Gold MassArray™ method (Sequenom Inc., San Diego, CA, USA) at the Spanish National Genotyping Center (Centro Nacional de Genotipado, Universitat Pompeu Fabra, Barcelona, Spain).

Statistical analysis

Differences between groups were assessed with the Student’s t-test (parametric) or the Mann–Whitney U test (non-parametric). Pearson or Spearman correlation coefficients were
used to evaluate the degree of association between variables. Each SNP was tested for Hardy–Weinberg equilibrium using Haploview 4.0 software [24]. Estimates of linkage disequilibrium between SNPs were calculated using Fisher’s test. PHASE (version 2) program with the SNPator package (http://www.snpator.com) were employed to determine the subsequent distribution of the haplotype frequencies within the study population. Haplotypic association analyses were also performed with the SNPator software. Diagnostic accuracy for serum PON1 lactonase and esterase activities and other biochemical tests was calculated with ROC analysis [25–27]. Multiple logistic regression models were fitted to estimate the ability of groups of variables to predict the presence or absence of disease. Statistical analyses were performed by biostatisticians (C.M-S. and A.N.) who were blinded with respect to the diagnostic groups. Results are shown as means and SD in parenthesis (parametric) or as medians and 95% CI (non-parametric). The SPSS 14.0 package was employed for all statistical calculations.

Results

Performance evaluation of serum lactonase activity

Linearity was assessed by triplicate measurements of serial dilutions of a sample with a high value (10.00 U/L). The regression line of observed vs. expected values was: \( y = 0.992 (0.007) x - 0.276 (0.180) \) \( r^2 = 0.981 (0.014); S_{y|x} = 0.940 (0.673); P < 0.001 \) (Supplementary Fig. 1). The method was linear between 0.62 and 10.00 U/L. To determine the detection limit, the absorbance of the reagent blank (buffer) was measured 20 times, the mean and standard deviation (SD) were calculated, and the detection limit was defined as the enzyme activity corresponding to an absorbance equal to the mean of the reagent blank value \( +3 \times SD \). The detection limit thus calculated was 0.12 U/L. Intra-assay imprecision was determined with 20 replicate analyses of the three pools performed in the same run. To assess inter-assay imprecision, aliquots of these pools stored at \(-40 \, ^\circ\text{C}\) were analyzed over 20 consecutive days. The coefficients of variation (CV) of the measurements ranged between 11.2 and 17.7% (Supplementary Table 1).

The interference from triglycerides, hemolysis, and jaundice was assessed by supplementing pools A, B, and C with chylomicrons, hemoglobin, or bilirubin at various concentrations [28,29]. The results showed that there were no substantial interferences by hemoglobin up to 6 g/L, bilirubin up to 300 \( \mu\text{mol/L} \), or triglycerides up to 15 mmol/L. (Supplementary Table 2).

Reference interval

Fig. 1 represents the frequency distributions histograms of serum lactonase and paraoxonase activities in our healthy population. The distributions of both variables were skewed. Reference intervals \((n=633)\) were the following: lactonase activity, median = 5.99 (95% CI = 3.29–13.61; mean = 6.78;
haplotype analysis are shown as Supplementary data. S, Ser), while those of the promoter region represent changes in the nucleotide sequence.

**Table 1** Distribution of PON1, PON2, and PON3 genotypes in the healthy population

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>dbSNP</th>
<th>Seattle SNP</th>
<th>Genotype frequency</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON1 rs662</td>
<td>18152</td>
<td>QQ/QR/RR &lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.51 0.41 0.08</td>
<td>Q/R 0.71 0.29</td>
</tr>
<tr>
<td>PON1 rs854560</td>
<td>9507</td>
<td>LL/LM/MM &lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.36 0.47 0.17</td>
<td>L/M 0.59 0.41</td>
</tr>
<tr>
<td>PON1 rs705381</td>
<td>1642</td>
<td>GG/GA/AA</td>
<td>0.59 0.36 0.05</td>
<td>G/A 0.77 0.23</td>
</tr>
<tr>
<td>PON1 rs854571</td>
<td>972</td>
<td>CC/CT/TT</td>
<td>0.18 0.42 0.06</td>
<td>C/T 0.73 0.27</td>
</tr>
<tr>
<td>PON1 rs854572</td>
<td>895</td>
<td>CC/GG/AA</td>
<td>0.20 0.53 0.17</td>
<td>C/G 0.59 0.41</td>
</tr>
<tr>
<td>PON1 rs854573</td>
<td>728</td>
<td>AA/GG/AA</td>
<td>0.59 0.36 0.05</td>
<td>A/G 0.77 0.23</td>
</tr>
<tr>
<td>PON1 rs757158</td>
<td>63</td>
<td>CC/CT/TT</td>
<td>0.39 0.47 0.14</td>
<td>C/T 0.62 0.38</td>
</tr>
<tr>
<td>PON3 rs7493</td>
<td>30199</td>
<td>SS/SC/CC &lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.62 0.34 0.04</td>
<td>S/C 0.79 0.21</td>
</tr>
<tr>
<td>PON3 rs12026</td>
<td>23956</td>
<td>AA/GG/AA</td>
<td>0.63 0.34 0.03</td>
<td>A/G 0.80 0.20</td>
</tr>
<tr>
<td>PON3 rs11760799</td>
<td>5913</td>
<td>CC/CT/TT</td>
<td>0.58 0.37 0.05</td>
<td>C/T 0.76 0.24</td>
</tr>
<tr>
<td>PON3 rs1770903</td>
<td>5815</td>
<td>AA/GG/AA</td>
<td>0.59 0.37 0.04</td>
<td>A/G 0.77 0.23</td>
</tr>
<tr>
<td>PON3 rs17882539</td>
<td>5734</td>
<td>CC/CT/TT</td>
<td>0.58 0.37 0.05</td>
<td>C/T 0.77 0.23</td>
</tr>
<tr>
<td>PON3 rs705381</td>
<td>2375</td>
<td>GG/GA/AA</td>
<td>0.62 0.34 0.04</td>
<td>G/A 0.79 0.21</td>
</tr>
<tr>
<td>PON3 rs1642</td>
<td>1510</td>
<td>TT/TT/GG</td>
<td>0.62 0.35 0.03</td>
<td>T/G 0.79 0.21</td>
</tr>
<tr>
<td>PON3 rs14984</td>
<td>1496</td>
<td>AA/GG/AA</td>
<td>0.62 0.34 0.04</td>
<td>A/G 0.79 0.21</td>
</tr>
</tbody>
</table>

<sup>a</sup> Abbreviations of the coding region polymorphisms represent changes in the protein amino acid sequence (A, Ala; C, Cys; G, Gly; L, Leu; M, Met; Q, Gln; R, Arg; S, Ser), while those of the promoter region represent changes in the nucleotide sequence.

**Discussion**

The synthesis of the TBBL substrate [30] has facilitated the development of a practical and rapid, high-throughput, semi-automated spectrophotometric assay for measuring serum PON1 lactonase activity. Specificity, kinetics and the effect of inhibitors have been previously reported [22,31]. The assay requires a low volume of sample (2 μL) which is important if the measurement is to be included in a battery of several tests, or

**Serum lactonase and paraoxonase activities as liver function tests**

Paraoxonase as well as lactonase showed progressive decreases depending on the severity of liver impairment; the values being lowest in patients with cirrhosis (Supplementary Table 3). There were weak, but statistically significant, correlations between serum lactonase activity and albumin concentration (r=0.21; P<0.001) and prothrombin time (r=0.37; P<0.001), as well as between serum paraoxonase activity and albumin concentration (r=0.23; P<0.001) and prothrombin time (r=0.35; P<0.001).

The results of the ROC analysis for lactonase and paraoxonase activities, as well as for the standard liver function tests, are shown in Table 3. The area-under-the-curve (AUC) for PON1 lactonase activity was similar to that of paraoxonase activity and close to 0.90 when comparing cirrhotic patients with healthy volunteers. The usefulness of adding serum PON1 lactonase and paraoxonase activities measurement to the standard panel of liver function tests was evaluated by multiple regression analysis. We compared the ability of two different models to correctly, and accurately, classify patients and controls. Overall, the addition of serum PON1 lactonase and paraoxonase activities measurement to the predictive models increased the diagnostic sensitivity to a similar extent as that of paraoxonase, without any impairment of specificity (Table 4). Both measurements were useful mainly in distinguishing between cirrhotic patients and healthy individuals.

The frequency distributions of the selected PON1, PON2, and PON3 polymorphisms in our healthy population are shown in Table 1. Bivariate statistical analysis showed that most of these polymorphisms were associated with changes in serum lactonase and paraoxonase activities, but the influence was consistently lower for serum lactonase (Table 2). PON1 rs662, PON1 rs11770903, PON3 rs854571, PON2 rs112026, and PON3 rs1642 polymorphisms showed the most prominent influence. The effect of PON1 rs662 polymorphism on paraoxonase activity was much higher than on lactonase. The maximum difference between the medians of the QQ and RR isoforms was 40.65% for the lactonase activity; with an increase of up to 167.82% for the paraoxonase activity.

Haplotype analysis defined 3 different blocks; the strongest influence being that of the block constituted by PON1 rs662 and PON1 rs11770903 polymorphisms. Again, the influence on esterase activity was higher than on lactonase activity. Results of the haplotype analysis are shown as Supplementary data.
when it is used for research purposes in experimental animals or in vitro cultures, where sample volume is a serious limitation. Imprecision was \(<18\%\), which is not too high for a non-commercial method in which the main reagent, TBBL, needs to be replenished at regular intervals. Linearity was excellent over the measurement range and the detection limit was sufficient for human and experimental animal studies. Lipidemia, jaundice and hemolysis appeared to have no influence on the assay, and which makes the measurement of lactonase activity reliable in patients with liver impairment. Using this method we have defined a PON1 lactonase activity reference range in a wide population sample representative of Reus (Catalunya) on the Spanish Mediterranean coast. The enzyme levels obtained were higher than those obtained in a study [22] conducted in healthy volunteers from Haifa, Israel (3.8±1.9 U/L; \(n=54\)). This study, in which the TBBL-based lactonase assay was initially described, had not attempted to establish population-based reference values, and we cannot be certain whether the differences observed are due to geographical or to genetic variability, or merely to chance.

<table>
<thead>
<tr>
<th>Polyorphism</th>
<th>Lactonase (U/L)(^a)</th>
<th>Dif. (%)(^b)</th>
<th>P-ANOVA</th>
<th>Paraoxonase (U/L)(^a)</th>
<th>Dif. (%)(^b)</th>
<th>P-ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON1(_{192})</td>
<td>QQ(^c) 5.39 (2.98−13.40)</td>
<td>40.44</td>
<td>&lt;0.001</td>
<td>240.54 (157.39−355.00)</td>
<td>167.82</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PON1(_{446})</td>
<td>MM(^c) 6.65 (4.42−14.36)</td>
<td>−34.13</td>
<td>&lt;0.001</td>
<td>644.21 (334.14−966.54)</td>
<td>−54.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PON1(_{1...162})</td>
<td>GG 5.73 (3.10−12.96)</td>
<td>14.65</td>
<td>0.002</td>
<td>298.70 (164.93−669.56)</td>
<td>8.80</td>
<td>0.688</td>
</tr>
<tr>
<td>PON1(_{099})</td>
<td>GG 6.70 (4.64−14.84)</td>
<td>−22.23</td>
<td>&lt;0.001</td>
<td>352.00 (198.63−790.83)</td>
<td>−27.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PON1(_{1076})</td>
<td>GG 6.57 (2.10−14.71)</td>
<td>−12.02</td>
<td>0.003</td>
<td>324.99 (195.84−724.84)</td>
<td>−7.58</td>
<td>0.718</td>
</tr>
<tr>
<td>PON1(_{1741})</td>
<td>CC 5.30 (2.89−12.09)</td>
<td>32.08</td>
<td>&lt;0.001</td>
<td>254.64 (155.38−548.80)</td>
<td>43.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PON2(_{311})</td>
<td>SS(^c) 5.69 (3.20−12.96)</td>
<td>36.20</td>
<td>&lt;0.001</td>
<td>283.17 (165.55−585.58)</td>
<td>58.30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PON2(_{448})</td>
<td>CC 7.75 (4.64−16.73)</td>
<td>−25.68</td>
<td>&lt;0.001</td>
<td>378.11 (178.69−666.85)</td>
<td>−24.81</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PON3(_{567})</td>
<td>CC 5.61 (3.37−13.92)</td>
<td>35.11</td>
<td>&lt;0.001</td>
<td>341.66 (174.57−700.56)</td>
<td>23.79</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PON3(_{465})</td>
<td>AA 3.69 (3.37−13.92)</td>
<td>35.11</td>
<td>&lt;0.001</td>
<td>341.00 (174.64−699.67)</td>
<td>23.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PON3(_{476})</td>
<td>AA 3.69 (3.37−13.92)</td>
<td>35.11</td>
<td>&lt;0.001</td>
<td>316.00 (168.98−716.60)</td>
<td>23.31</td>
<td>&lt;0.001</td>
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<td>PON3(_{4105})</td>
<td>AA 7.75 (4.64−16.64)</td>
<td>−25.68</td>
<td>&lt;0.001</td>
<td>340.33 (174.57−700.56)</td>
<td>23.31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PON3(_{4970})</td>
<td>GG 5.69 (3.20−12.96)</td>
<td>−26.58</td>
<td>&lt;0.001</td>
<td>283.17 (165.55−585.58)</td>
<td>46.50</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\(^a\) Serum enzyme activities are shown as medians and 95% CI.

\(^b\) Dif. represents the difference, expressed as a percentage of increase or decrease, observed between the medians of the enzyme activities corresponding to the two homozygous forms of every SNP.

\(^c\) Abbreviations of the coding region polymorphisms represent changes in the protein amino acid sequence (A, Ala; C, Cys; G, Gly; L, Leu; M, Met; Q, Gln; R, Arg; S, Ser), while those of the promoter region represent changes in the nucleotide sequence.
Serum lactonase activity was significantly correlated with paraoxonase activity. However, this relationship depended on the PON1<sub>192</sub> polymorphism; the correlation coefficient being higher for the QQ homozygous than for carriers of the R allele. In this respect, the TBBL assay is similar to other PON1 esterase assays. For example, Furlong et al. [32] recently reviewed their studies using a two-substrate approach to examine the PON1 activity towards paraoxon as compared to the ability of PON1 to hydrolyse other substrates such as chlorpyrifos oxon, diazoxon, phenyl acetate, soman, and sarin. In all these cases, when the enzyme activities were plotted against paraoxonase activity, trimodal distributions were consistently observed, and reflected PON1<sub>192</sub> polymorphism with a degree of overlapping between carriers of the different PON1<sub>192</sub> alleles (QQ, QR and RR). This observation highlights the relevance of genetic variability in determining the circulating PON1 levels, and poses a problem for the proposal of paraoxon-measured serum PON1 activity as a candidate for inclusion in the battery of liver function tests i.e. the interpretation of variation in levels of any disease marker is more difficult when a genetic influence is high. We compared the influence of selected polymorphisms of PON1, PON2, and PON3 genes on lactonase as well as paraoxonase activities of PON1. Serum lactonase activity is much less influenced by the PON1<sub>192</sub> polymorphism than is the paraoxonase activity. While paraoxonase activity in RR carriers can be 2–3 times higher than that of the QQ carriers, we observed only a 30–50% increase in lactonase activity associated with the polymorphism. The influence of the other polymorphisms studied on the esterase activity of PON1 was similar to that reported in previous studies [33–36] and somewhat higher than on the lactonase activity. Taken together, the genotype–phenotype studies showed that serum lactonase activity is considerably less influenced by PON1 polymorphisms than the polymorphism’s influence on the enzyme’s paraoxonase activity.

A secondary goal of the present investigation was to describe the genotype and allele frequencies of selected PON1, PON2, and PON3 gene polymorphisms in a large sample (n=633) of healthy Mediterranean Caucasian volunteers involved in a population-based study being conducted in our research unit. The genotype and allele frequencies of PON1 and PON2 SNP’s we observed were similar to those reported in other Caucasian populations in Europe and the USA [34,37] and similar as well to Pakistani [38], Asian Indians [39], and Mexican [35] populations. We report, for the first time to the best of our knowledge in a healthy population, the frequencies of the PON1<sub>1076</sub> polymorphism which is a genetic variant that had been described recently in patients with diabetes mellitus, and with frequencies practically identical to ours [40]. PON1<sub>1076</sub> SNP may have clinical relevance because it was associated with enhanced albuminuria in diabetic patients. We report, as well, the genotype frequencies of six new PON3 promoter SNP’s, which showed a strong linkage between themselves and with PON1 and PON2 polymorphisms.

Our data show that the measurement of serum PON1 lactonase activity is as efficient as paraoxonase, in terms of diagnostic accuracy, in testing for liver dysfunction. Clinical diagnosis of chronic liver impairment and/or liver fibrosis is currently conducted via the invasive procedure of needle biopsy followed by the histological evaluation of the material on a numerical scoring system. This procedure has important drawbacks, including a significant mortality rate (1/1000–1/10,000), sampling error, and subjectivity of the histological evaluation. Therefore, the development of non-invasive tests for diagnosis was an issue of great importance. In this respect, the lactonase activity calculated from the equation is

\[ L = 0.038 \times \text{PT} + 9.678 \]

A secondary goal of the present investigation was to describe the genotype and allele frequencies of selected PON1, PON2, and PON3 gene polymorphisms in a large sample (n=633) of healthy Mediterranean Caucasian volunteers involved in a population-based study being conducted in our research unit. The genotype and allele frequencies of PON1 and PON2 SNP’s we observed were similar to those reported in other Caucasian populations in Europe and the USA [34,37] and similar as well to Pakistani [38], Asian Indians [39], and Mexican [35] populations. We report, for the first time to the best of our knowledge in a healthy population, the frequencies of the PON1<sub>1076</sub> polymorphism which is a genetic variant that had been described recently in patients with diabetes mellitus, and with frequencies practically identical to ours [40]. PON1<sub>1076</sub> SNP may have clinical relevance because it was associated with enhanced albuminuria in diabetic patients. We report, as well, the genotype frequencies of six new PON3 promoter SNP’s, which showed a strong linkage between themselves and with PON1 and PON2 polymorphisms.

Our data show that the measurement of serum PON1 lactonase activity is as efficient as paraoxonase, in terms of diagnostic accuracy, in testing for liver dysfunction. Clinical diagnosis of chronic liver impairment and/or liver fibrosis is currently conducted via the invasive procedure of needle biopsy followed by the histological evaluation of the material on a numerical scoring system. This procedure has important drawbacks, including a significant mortality rate (1/1000–1/10,000), sampling error, and subjectivity of the histological evaluation. Therefore, the development of non-invasive tests for diagnosis was an issue of great importance. In this respect, the lactonase activity calculated from the equation is

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the diagnosis of liver disease and the extent of the disease is an important goal of current research [41,42]. Unfortunately, most of the individual laboratory tests to assess liver impairment have low specificity and sensitivity and, hence, the standard approach is to perform a battery of several tests followed by an algorithmic evaluation of the results. It is for this reason that several years ago we proposed the addition of serum PON1 paraoxonase activity measurement as a biomarker of liver impairment [19,21]. Serum paraoxonase-1 measurement has an important feature in that the measured value is inversely related to the degree of liver derangement i.e. it decreases while most of the standard laboratory test values increase with the extent of the disease. Thus, paraoxonase measurement has an additional contribution in improving current algorithms, and the ratios between tests. We estimated, by multiple logistic regression analysis, that the addition of paraoxonase measurement to a battery of standard liver function tests increased the overall sensitivity up to ≥90%, while keeping the specificity close to 100%. However, the two important constraints on the use of the paraoxon method in clinical practice are the extremely high toxicity of paraoxon and the strong influence of PON1 gene polymorphisms on the enzyme’s activity. The measurement of the lactonase activity of PON1 has not these drawbacks. TBBL substrate is not toxic, and the influence of genetic polymorphisms, especially of PON1192, is much lower. Of note in the present study is that lactonase diagnostic accuracy is no higher than that of paraoxonase, despite showing a lower influence of the genetic background. This is probably because, in patients with liver impairment, the percentage decreases in the lactonase assay values are lower than that of the paraoxonase values. For example, patients with liver cirrhosis have about a 50% decrease in serum lactonase activity and a 67% in paraoxonase relative to non-cirrhotic patients. Despite the inconveniences inherent in both methods, the equivalence of both tests in terms of diagnostic accuracy but with the better safety of TBBL substrate for the laboratory worker, makes the lactonase measurement a strong candidate for inclusion into the routine clinical laboratory testing of liver impairment, or for the study of other diseases involving oxidative stress.

In conclusion, in the present study we report a fast, reliable semi-automated assay for the measurement of serum PON1 lactonase activity based on a non-toxic substrate. We studied the influence of genetic polymorphisms of PON on the lactonase activity, and which was considerably lower than of paraoxonase activity. Based on our findings, we suggest that serum lactonase activity measurement may contribute significantly to the evaluation of liver function in the clinical setting.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clinbiochem.2008.09.120.

References


